USP8 Mutations and Cell Cycle Regulation in Corticotroph Adenomas

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ABSTRACT

Corticotroph adenomas frequently harbor somatic *USP8* mutations. These adenomas also commonly exhibit underexpression of P27, a cell cycle regulator. The present study aimed to determine the influence of *USP8* mutations on clinical features of Cushing's disease and to elucidate the relationship between *USP8* mutations and P27 underexpression in these tumors. Retrospective study with 32 patients with Cushing's disease was followed at the Ribeirao Preto Medical School University Hospital. We evaluated the patients' clinical data, the *USP8* mutation status and the gene expression of cell cycle regulators *P27/CDKN1B*, *CCNE1*, *CCND1*, *CDK2*, *CDK4,* and *CDK6* in tumor tissue in addition to the protein expression of P27/CDKN1B. We observed somatic mutations in the exon 14 of *USP8* in 31.3 % of the patients. Larger tumor size was observed in patients harboring *USP8* mutations ($p = 0.04$), with similar rates of remission, age of presentation, salivary cortisol at 23:00 h and after 1 mg dexamethasone, ACTH levels, and early post-operative plasma cortisol. We observed no differences regarding the gene or protein expression of the cell cycle regulators according to *USP8* mutation status. In this Brazilian series, the observed frequency of *USP8* somatic mutations was similar to that reported in European ancestry populations. Although it was reasonable that *USP8* mutations could contribute to cell cycle dysregulation and P27 underexpression in corticotroph adenomas, our data did not confirm this hypothesis. It is possible that increased deubiquitinase activity observed in mutated *USP8* might influence other pathways related to cell growth and proliferation.

Introduction

The cell-cycle progression is synchronized by cyclins and cyclin-dependent kinases (CDKs) [1]. The misexpression of these cell cycle regulators has been associated with pituitary tumorigenesis, in animal models [2, 3] and in human pituitary adenomas [1, 4]. Notably, the underexpression of P27 or CDKN1B [cyclin-dependent kinase (CDK) inhibitor 1B] has been observed in all types of sporadic pituitary adenomas, mainly in corticotroph adenomas [5–8].

Amongst the studied mechanisms regulating P27 protein expression in sporadic pituitary corticotroph adenomas, *CDKN1B* mutations [9–11], promoter hypermethylation [12], or impaired translation by the misexpression of regulatory microRNAs [13] have not been frequently observed. On the other hand, P27-increased ubiquitination, phosphorylation, and subsequent degradation may account for reducing P27 expression [14–17]. Another potential mechanism that could partially explain the P27 underexpression in corticotroph adenomas is the loss of CABLES1 expression [18], also a cell cycle regulator. However, somatic *CABLES1* mutations were infrequent events in a large cohort of Cushing's disease patients [19].

Although extensive evaluation of candidate genes largely failed to identify somatic mutations underlying corticotroph tumorigenesis, recent studies using next-generation sequencing shed light on this issue with the discovery of somatic mutations in the exon

14 of *USP8* (ubiquitin-specific protease 8) in 35–60 % of corticotroph adenomas [20, 21], while *BRAF* and *USP48* mutations were observed in corticotroph adenomas carrying wild-type *USP8* [22]. The *USP8* gene encodes for a deubiquitinase that inhibits the lysosomal degradation of epidermal growth factor receptor (EGFR) [23]. The mutant USP8 loses the ability to bind to 14-3-3 proteins, which increases USP8 proteolytic cleavage. The cleaved fragment exhibits higher deubiquitinase activity [20], resulting in increase of EGFR, which overexpression had been previously described in these adenomas [24, 25].

P27 is a target of EGFR signaling in different cell models [26]. In human cell lines derived from estrogen receptor-alpha-negative invasive breast carcinoma, EGF treatment and EGFR phosphorylation cause nuclear translocation of JAB1 and a consequent decrease in P27 expression [27]. It has also been demonstrated that the E5 oncoprotein from the human papillomavirus type 16 promotes downregulation of P27 through EGFR signaling [28]. In corticotroph adenomas, low P27 protein expression inversely correlates with EGFR levels [24]. Considering that *USP8* mutation activates EGFR-MAPK signaling, we speculate that *USP8* mutations could induce P27 underexpression in corticotroph adenomas.

In order to further clarify the relationship between *USP8* mutation and cell cycle dysregulation in corticotroph adenomas, we evaluated the clinical data of a series of patients with Cushing's disease, assessed their *USP8* mutation status, determined the impact of *USP8* mutations on the clinical features of Cushing's disease, verified the protein expression of P27 and the gene expression of *CDKN1B*, *CCNE1*, *CCND1*, *CDK2*, *CDK4*, and *CDK6*, crucial regulators of cell cycle.

Subjects and Methods

Subjects

We performed a retrospective study with 32 patients (31 females/1 male) who underwent transsphenoidal surgery for Cushing's disease (CD). All patients were followed in the Endocrine Center in the Division of Endocrinology, University Hospital of the Ribeirao Preto Medical School, University of Sao Paulo, one of the referral services for Cushing's syndrome in Brazil. This study was approved by the Institutional Ethical Committee (Process no 11071/2013) and written informed consent was obtained from all subjects. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Cushing's syndrome diagnosis was established on clinical assessment and biochemical evidence of hypercortisolism, according to international criteria [29]. Briefly, biochemical confirmation was based on increased levels of urinary free cortisol and/or late-night salivary cortisol (LNSC), and non-suppression of plasma or salivary cortisol after low-dose (1 mg overnight) dexamethasone suppression test (LDDST). Plasma and salivary cortisol were measured by radioimmunoassay (RIA), as previously described [30] and to exclude the biochemical diagnosis of endogenous hypercortisolism, we used as cut off values for LNSC values lower than 350 ng/dl (or 9.8 nmol/l) and salivary cortisol levels after LDDST lower than 150 ng/dl (or 4.2 nmol/l) [31]. After the confirmation of hypercortisolism, ACTH dependency was determined by normal or increased basal plasma ACTH (ACTH above 2.2 pmol/l or 10 pg/ml).

The differential diagnosis between pituitary and ectopic etiologies was performed by dynamic tests [32]. For CD, a suppression of plasma cortisol above 50 % after high-dose (8 mg overnight) dexamethasone suppression test (HDDST) was considered and, depending on availability, CRH-test (cut-off value: increase above baseline of 35 % in ACTH and of 20 % in cortisol) or DDAVP test (cut-off value: increase above baseline of 50 % in ACTH and of 20 % in cortisol) was performed. In the case of concordant dynamic biochemical studies, patient underwent pituitary imaging. If the patient presented a focal lesion (larger than 5 mm in diameter) on pituitary MRI, CD was diagnosed and no further evaluation was performed. In the case of discordant or equivocal biochemical or radiological studies, bilateral inferior petrosal sinus sampling (BIPSS) with CRH or DDAVP stimulation was performed and considered consistent with CD with an inferior petrosal sinus to peripheral ACTH ratio (IPS/P) greater than 3.0 [33]. In addition, familial history of pituitary adenomas or endocrine tumors was carefully assessed and systematic measurement of levels of calcium was performed to exclude coexistent hyperparathyroidism.

All patients underwent transsphenoidal adenomectomy and surgical remission was defined as early postoperative concentrations of morning plasma cortisol below 3 µg/dl (85 nmol/l). The tumor samples were collected during surgery. Part of the tissue was separated for routine histopathological examination and part was immediately frozen and microdissected to isolate tumoral tissue. The microdissected sample was mechanically disrupted and stored at −70 °C for molecular studies. The control group for gene expression experiments included seven normal pituitary (NP) tissues obtained during autopsies as previously described [13]. Furthermore, hematoxylin-eosin staining was performed to confirm the presence of the tumor or to exclude incidental pituitary adenoma in NP.

Nucleic acid extraction

DNA and RNA were isolated by TRIzol® reagent (Invitrogen/Thermo Fisher Scientific, Carlsbad, CA, USA). Sample integrity was evaluated by spectrophotometry at an absorbance of 260/280 nm using NanoDrop™ 2000/2000c (Thermo Fisher Scientific, Wilmington, Delaware, USA) and by agarose gel electrophoresis.

USP8 mutation screening

The exon 14 of the *USP8* gene was amplified by PCR using primers previously described [34]. The amplified products were sequenced using a commercial kit (Big Dye™ Terminator Cycle Sequencing; Applied Biosystems/Thermo Fisher Scientific, Foster City, CA, USA). The acquired sequences were compared to *USP8* reference sequence (GenBank NM_005154.4) using the CodonCode Aligner Program V4.0.4 (CodonCode Co.).

Reverse transcription PCR and real-time PCR quantification

From 32 tumor samples submitted to *USP8* sequencing by Sanger method, RNA was available in a subset of 21 tumor samples. In these samples, cDNA was obtained using High Capacity cDNA Reverse Transcription kit (Applied Biosystems/Thermo Fisher Scientific, Foster City, CA, USA). To evaluate the expression of genes involved in the cell cycle control, qPCR was performed by 7500 RT-PCR System (Applied Biosystems/ Thermo Fisher Scientific, Foster City, CA, USA) using TaqMan® Gene Expression Assays: *CDKN1B* (Hs01597588_m1), *CCNE1* (Hs01026536_m1), *CCND1* (Hs00277039_m1), *CDK2* (Hs01548894_m1), *CDK4* (Hs00175935_m1), *CDK6* (Hs01026371_m1) and reference genes: *PGK1* (4326318E), *TBP* (4326322E) and *GUSB* (4326320E). Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method, as previously described [13, 35].

Immunohistochemistry (IHC)

IHC anti-P27 was performed in all tumor samples. The paraffin-embedded samples underwent deparaffinization, rehydrataion, antigen retrieval (citrate buffer pH 6.0 for 40 min at 100 °C), peroxide block, rinse, protein block, rinse, and incubation with 1:200 P27/CDKN1B primary antibody (SAB4300420) (Sigma-Aldrich, St Louis, MO, USA) overnight at room temperature. The signal was amplified with the horseradish peroxidase polymer method (MACH1 Universal HRP-Polymer Detection, Biocare Medical, Concord, CA, USA). The chromogen used was DAB and the counterstaining was performed with hematoxylin. Normal pituitary tissue was used as positive control.

Each sample was assigned into two categories according to the following score: category A documented the number of immunoreactive cells as 0 (< 10 %), 1 (≥ 10 < 50 %), and 2 (≥ 50 %); category B documented the intensity of the immunostaining as 1 (no or weak) and 2 (moderate or strong). The values for categories A and B were added to provide the immunoreactivity score, ranging from 1 to 4 (scores 1, 2 = low expression; score 3 = moderate expression; and score 4 = high expression). The stained tumor tissues were scored while blinded to the clinical or molecular data.

Statistical analysis

Data are presented as mean ± standard deviation, median and interquartile range. Mann–Whitney tests were used for continuous variables and Fisher's exact tests for categorical variables. Differences were considered significant at $p < 0.05$. Data were analyzed by GraphPad Prism 5 software.

Results

Individual clinical, laboratorial, and molecular data of the 32 studied patients can be seen in ▶ **Table 1**. As expected for studies evaluating Cushing's disease in adults, females were more represented than males and the majority of the patients presented microadenomas [36]. Twenty-four out of the 32 patients exhibited microadenomas and the tumors did not present cavernous sinus invasion. All patients presented high levels of LNSC (85.4 ± 80.9 nmol/l, range 23.1–497.4), no salivary cortisol suppression after LDDST (70.0 ± 50.3 nmol/l, range 9.1–222.6), and high ACTH levels (18.5 ± 9.3 pmol/l, range 7.8–42.0). After HDDST, we observed plasma cortisol suppression of 68.6 ± 27.0 %. Regarding ACTH increment after CRH or DDAVP stimulation tests, we observed 103.6 ± 89.8 and 125.8 ± 148.1 % of increment, respectively. Fourteen out of 32 patients were submitted to BIPSS and in all of them the IPS/P gradient confirmed the pituitary etiology. The majority of patients (29/32) underwent transsphenoidal surgery as first choice therapy; two patients were preoperatively treated with ketoconazole, and one with bilateral adrenalectomy due to the severity of the disease. After transsphenoidal surgery, the remission rate was 65.6 %; patients with persistent disease were submitted to complementary therapy with a second transsphenoidal surgery, or ketoconazole, or adrenalectomy, or radiotherapy, or combination of them.

USP8 mutations and clinical phenotype

In 10 out of the 32 tumors (31.3 %) we observed mutations in the exon 14 of *USP8*. The mutation c.C2159G (p.P720R) was observed in 5 patients, the c.2151_2153delCTC (p.S718del) in 3 patients, while 2 other mutations were observed each in one patient: c.T2152C (p.S718P) and c.2155_2169delTCCCCAGATATAACC (p.S719_T723del). All these mutations have been previously described [20, 21, 34].

Patients harboring somatic *USP8* mutations exhibited similar age of presentation than patients without *USP8* mutations. They also presented similar late night salivary cortisol, salivary cortisol after LDDST, ACTH levels, and postoperative plasma cortisol levels. We observed superior tumor size in patients harboring *USP8* mutations ($p = 0.04$). Both groups had similar rates of remission (▶ **Table 2**).

Gene expression

We performed Real Time PCR in samples obtained from 21 patients (7 of them harboring *USP8* mutations). We observed no differences regarding the gene expression of the cell cycle regulators *CDKN1B (P27)*, *CCNE1 (CYCLIN-E1)*, *CCND1 (CYCLIN-D1)*, *CDK2*, *CDK4*, and *CDK6* according to *USP8* mutation status (▶ **Table 3**).

Immunohistochemistry

P27 (CDKN1B) was expressed in 31 pituitary adenomas. In one case, there was no sufficient tissue for IHC analysis. ▶ **Fig. 1** shows representative examples of photomicrographs of the immunohistochemical analysis. In tumors harboring the *USP8* mutation, low to moderate positivity was observed in 6 (66.7 %), and high positivity in 3 (33.3 %) of the analyzed adenomas. Similarly, in tumors without the *USP8* mutation, low to moderate positivity was observed in 14 (63.6 %), and high positivity in 8 (36.3 %) of the analyzed adenomas ($p = 1.0$). Regarding the staining pattern, in the tumors harboring the *USP8* mutation, we observed predominant nuclear in only one tumor (11.1 %), predominant cytoplasm in 3 (33.3 %), and only cytoplasm in 5 (55.6 %). In the tumors without the mutation, the staining pattern was predominant nuclear in 6 (27.3 %), predominant cytoplasm in 10 (45.4 %), and only cytoplasm in 6 (27.3 %) ($p = 0.6$).

Discussion

In the present study, we detected *USP8* somatic mutations in 31.3 % of Brazilian patients with Cushing's disease. Patients harboring somatic *USP8* mutations exhibited superior tumor size but similar clinical and biochemical phenotype, and similar pattern of expression of cell cycle regulators suggesting that *USP8* mutations might not be implicated in cell cycle dysregulation in corticotroph adenomas.

▶ **Table 1** Clinical and laboratorial features of Cushing's disease patients.

Patient	*USP8* mutation status	Age at diagnosis (years)	Sex	LNSC (nmol/l)	Salivary cortisol post LDDST (nmol/l)	ACTH (pmol/l)	Tumor Size (cm) (MRI)	IHC
1	WT	31	M	85.12	NA	33.9	after Adx - 0.9	ACTH +
2	Del c.2155_2169del TCCCCAGA-TATAACC (p.S719_T723del)	45	F	63.42	64.68	16.3	1.0	ACTH+, CAM.2+
3	Del c.2151_2153delCTC (p.S718del)	26	F	47.6	112.0	28.4	0.8	ACTH+
4	WT	54	F	46.06	70.0	11.7	0.3	ACTH+, ki67 < 1 %
5	c.T2152C (p.S718P)	38	F	145.32	81.48	18.4	no tumor	negative
6	WT	26	F	94.08	73.92	13.0	no tumor	ACTH+
7	WT	39	F	57.82	112.0	18.5	0.3	inconclusive
8	WT	11	F	131.6	103.32	16.6	0.4	ACTH+
9	WT	36	F	57.68	94.5	10.8	2.0	ACTH+
10	WT	64	F	48.44	30.8	30.6	0.5	ACTH+, focal Gal-3
11	WT	19	F	94.08	81.48	11.3	0.5	inconclusive
12	WT	23	F	89.6	9.1	11.3	0.4	ACTH+
13	Del c.2151_2153delCTC(p.S718del)	32	F	86.8	100.8	25.8	0.6	ACTH+
14	c.C2159G (p.P720R)	17	F	109.2	44.8	11.1	3.6	ACTH +
15	WT	34	F	69.72	222.6	42.0	0.4	ACTH+
16	WT	35	F	68.6	47.32	41.6	0.6	ACTH+, invasion in neurohypophysis
17	WT	33	F	39.2	21.28	15.8	0.3	ACTH+
18	WT	31	F	68.46	26.32	23.3	0.9	ACTH+
19	c.C2159G (p.P720R)	21	F	102.2	45.92	7.8	1.2	ACTH+
20	c.C2159G (p.P720R)	24	F	23.1	32.62	7.8	1.9	ACTH+, MIB 1 8 %
21	Del c.2151_2153delCTC (p.S718del)	27	F	54.88	20.16	21.0	0.5	ACTH+
22	WT	35	F	37.38	43.68	15.5	0.3	ACTH+
23	WT	47	F	35.56	61.74	19.9	0.2	ACTH+
24	WT	29	F	106.4	112.0	20.2	0.5	hyperplasia
25	WT	14	F	112.0	112.0	10.2	0.6	ACTH+
26	WT	43	F	36.68	35.84	11.3	0.5	ACTH+
27	c.C2159G (p.P720R)	23	F	43.54	31.78	7.9	0.5	negative
28	WT	31	F	52.22	70.98	26.0	2.0	ACTH+, ki67 3 % (Crooke)
29	WT	47	F	497.42	209.44	28.2	1.2	ACTH+/Crooke
30	WT	27	F	89.6	36.68	10.8	0.6	ACTH and GH+
31	WT	49	F	48.3	14.84	14.0	0.5	inconclusive
32	c.C2159G (p.P720R)	44	F	91.0	47.6	12.9	1.0	ACTH+

LNSC: Late-night salivar cortisol; LDDST: Low-dose (1 mg overnight), dexamethasone supression test; MRI: Magnetic ressonance imaging; IHC: Immunohistochemistry; WT: Wild type, F: Female; M: Male, NA: Not available, Adx: Adrenalectomy. a: Patient submitted to Liddle 1 test. Patients 4, 5, 6, 12, 15, 16, 17, 21, 22, 23, 24, 25, 26, and 31 were submitted to bilateral inferior petrosal sinus sampling.

The observed frequency of *USP8* mutations in this study (31.3 %) is in agreement with previous worldwide series, reporting *USP8* mutations in 35–60 % of corticotroph adenomas [20, 21, 34, 37, 38]. The age of presentation, LNSC, salivary cortisol after LDDST, ACTH levels, and rates of remission of our patients harboring *USP8* mutations were similar to the patients without mutations, reinforcing the scant data available on this issue [21, 34, 37]. In our series, comprised majority by women, a larger tumor size in patients harboring *USP8* mutations was observed. In accordance with our data, a European study observed that tumors with *USP8* mutations were slightly larger than wild-type tumors in female patients [34], whereas studies evaluating Asiatic populations observed inferior tumor size in the presence of *USP8* mutations [21, 37]. These differences could be potentially ascribed to different genetic backgrounds related to ethnicity. Of note, our patients are from the Brazilian Southeast, whose population background has been shaped by immigrants from several countries of Europe, including Italy, Spain, and Germany [39]. In addition, it is not possible to rule out an effect of estrogens on *USP8* mutant corticotroph cells [34, 40].

P27 underexpression has been extensively demonstrated in different series of pituitary adenomas [5–8]. Recently, our lab also demonstrated P27 nuclear underexpression, even in tumors exhibiting *CDKN1B* mRNA normally expressed, suggesting that post-transcriptional mechanisms could underlie P27 underexpression in pituitary adenomas. However, we showed that P27 aberrant expression in pituitary tumors could not be explained by dysregulation of several *CDKN1B* translational controllers (DKC1, RPS13, *miR221*, *miR222*) or by *DKC1* mutations [13]. In the present study, we speculated that *USP8* exon 14 mutations could promote P27 insufficiency and consequential cell cycle dysfunction via EGR pathway activation in corticotroph adenomas.

Our findings on gene expression of *CDKN1B (P27)*, *CCNE1*, *CCND1*, *CDK2*, *CDK4*, and *CDK6* showed no significant differences between WT and *USP8* mutant groups. In addition, based on our mechanistic hypothesis, we evaluated cell cycle regulators not only at transcript level, but also the P27/CDKN1B protein. We found no significant difference in P27 protein expression between WT and *USP8* mutant groups, neither in immunoreactivity score nor in cell compartment distribution, suggesting no relationship between cell cycle regulators and *USP8* mutations in corticotroph adenomas.

It has been previously shown that corticotroph adenomas harboring *USP8* mutations display higher EGFR expression [21] and AtT20 cells treated with USP8 inhibitor exhibited downregulation of EGFR expression [41]. However, Hayashi et al. observed no significant differences in EGFR mRNA or IHC expression between *USP8* WT and mutant adenomas [37]. In accordance, Ballmann et al. observed EGFR negative IHC staining in *USP8* mutant adenomas [42]. In vitro functional analysis of *USP8* mutations revealed increased deubiquitinase activity, which inhibits the downregulation of EGFR, but this finding was not replicated in vivo [37]. Some of these differences could be ascribed to the use of different antibodies against the EGFR [43].

One of the main strengths of this single center study was the homogeneity of the diagnostic and postoperative management protocols, allowing a comprehensive phenotypic characterization. The limitation was the relatively small number of samples carrying *USP8* somatic mutations in our series. Nonetheless, this study originally demonstrated that the aberrant expression of P27 in corticotroph adenomas is unrelated to *USP8* mutations. Hence, additional studies are required to elucidate the role of *USP8* mutations in Cushing's disease to adequately determine suitable patients for directed therapies.

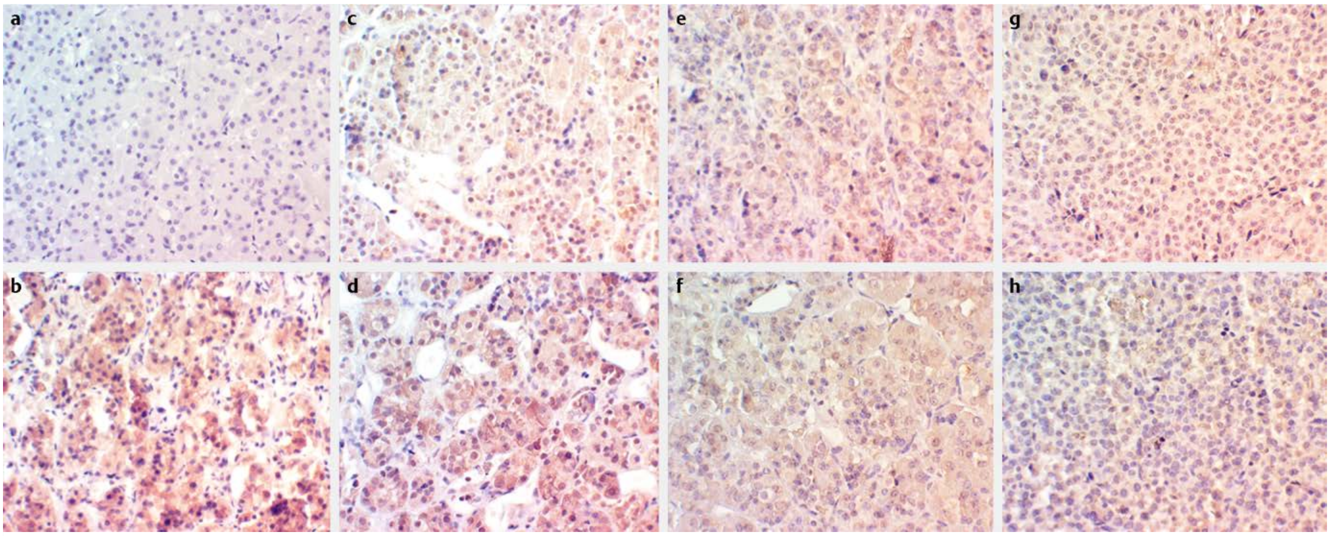
▶ **Table 2** Clinical features of Cushing's disease patients according to *USP8* mutation status.

	***USP8* mutated tumors (n = 10)**	***USP8* wild-type (n = 22)**	**p**
Age of presentation (years)	29.7 ± 9.7 26.5 [22.5–39.5]	34.4 ± 12.7 33.5 [26.75–44.0]	0.3
Late-night salivary cortisol (nmol/l)	76.7 ± 36.8 75.1 [46.6–104.0]	89.3 ± 95.1 68.5 [47.7–94.1]	0.84
Salivary cortisol post LDDST (nmol/l)	58.2 ± 30.8 46.8 [32.4–86.3]	75.7 ± 57.1 70.0 [33.3–107.7]	0.62
ACTH (pmol/l)	15.7 ± 7.5 14.6 [7.9–22.2]	19.8 ± 9.9 16.2 [11.3–26.5]	0.26
Maximum tumor diameter (mm)	11.1 ± 10.1 9 [5–13.7]	5.7 ± 5.4 5 [3–6]	0.04
Postoperative plasma cortisol (nmol/l)	166.3 ± 177.3 45.5 [33.0–346.2]	84.6 ± 112.3 33.0 [33.0–106.3]	0.20
Remission	40 % (4/10)	63.6 % (14/22)	0.27

Data expressed as mean ± standard deviation, median [interquartile range], except for remission. LDDST: Low-dose dexamethasone suppression test.

▶ **Table 3** Relative gene expression of cell cycle regulators in corticotroph adenomas according to *USP8* mutation status.

Gene	***USP8* mutated Tumors (Relative Expression $2^{-\Delta\Delta Ct}$) mean ± SD median [Q2–Q4]**	***USP8* non-mutated Tumors (Relative Expression $2^{-\Delta\Delta Ct}$) mean ± SD median [Q2–Q4]**	**p**
CDKN1B (*P27*)	0.85 ± 0.91 0.35 [0.08–1.65]	1.34 ± 1.03 1.13 [0.60–2.19]	0.3
CDK2	2.31 ± 1.70	2.09 ± 1.22	0.96
	1.53 [0.88–3.70]	1.91 [1.02–3.20]	
CDK4	0.60 ± 0.25	0.90 ± 0.80	0.76
	0.72 [0.35–0.78]	0.63 [0.39–0.94]	
CDK6	0.44 ± 0.52	0.72 ± 0.62	0.16
	0.08 [0.02–1.02]	0.48 [0.16–1.32]	
CCNE1	2.10 ± 2.26	1.74 ± 1.53	0.67
(*CYCLIN E1*)	1.40 [0.70–1.98]	1.05 [0.74–2.47]	
CCND1	0.60 ± 0.37	0.93 ± 0.66	0.34
(*CYCLIN D1*)	0.55 [0.26–0.90]	0.70 [0.48–1.25]	



► **Fig. 1** Representative photomicrographs of immunohistochemical analysis: Negative control of P27/CDKN1B immunohistochemistry, with no primary antibody added, in a specimen of normal pituitary (×400) **a**. Positive control of P27/CDKN1B immunohistochemistry in a specimen of normal pituitary (×400) **b**. Histologic specimens of *USP8* mutated ACTH-secreting pituitary adenomas (×400) with P27/CDKN1B immunohistochemistry showing moderate positive score and predominant nuclear staining **c**; moderate positive score and predominant cytoplasm staining **e**; and low positive score and predominant cytoplasm staining **g**. Histologic specimens of *USP8* wild type ACTH-secreting pituitary adenomas (×400) with P27/CDKN1B immunohistochemistry showing strong positive score and predominant nuclear staining **d**; moderate positive score and predominant cytoplasm staining **f**; and low positive score and predominant cytoplasm staining **h**.

In conclusion, in this Brazilian series of patients with Cushing's disease, the observed frequency of *USP8* somatic mutations was similar to that reported in European ancestry populations. Although it was reasonable that *USP8* mutations could contribute to cell cycle dysregulation and P27 underexpression in corticotroph adenomas, our data did not confirm this hypothesis. It is possible that increased deubiquitinase activity observed in mutated *USP8* corticotroph adenomas might influence P27 post-translational phosphorylation or other pathways involved in cell growth and proliferation. Furthermore, other phenomena, unrelated to *USP8* mutations may also regulate P27 underexpression in corticotroph adenomas.

Author Contributions

C.S.M. and M.C. planned the study, analyzed data, and wrote the manuscript. C.S.M. and R.C.C. performed gene expression studies. C.S.M and F.B.C.L performed genetic analysis. F.P.S. performed the macrodissection of histopathological specimens and the IHC analysis. C.S.M. collected clinical data and performed statistical analysis. A.C.M contributed with critical analysis of the data. All authors reviewed the manuscript.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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